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The Effect of Bronchoconstriction by Methacholine Inhalation in a Murine Model of Asthma

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Keywords
Asthma model · Airway remodeling · Bronchoconstriction · Mechanical stress · Methacholine

Abstract
Introduction: Bronchoconstriction was recently shown to cause airway remodeling and induce allergic airway inflammation in asthma. However, the mechanisms how mechanical stress via bronchoconstriction could induce airway inflammation and remodeling remain unclear. ***Objective:*** We investigated the effect of bronchoconstriction induced by methacholine inhalation in a murine model of asthma. ***Methods:*** BALB/c female mice were sensitized and challenged with ovalbumin (OVA), followed by treatment with methacholine by a nebulizer twice a day for 7 days. Twenty-four hours after the last methacholine treatment, the bronchoalveolar lavage fluid (BALF) and lung tissues were collected. The BALF was analyzed for total and differential cell counts and cytokine levels. The lung tissues were analyzed for goblet cell metaplasia, thickness of the smooth muscle, and lung fibrosis. The expression of cytokines in the lung was also examined. ***Results:*** OVA sensitization and challenge induced infiltration of total cells, macrophages, and eosinophils in the BALF along with goblet cell metaplasia and increased airway smooth muscle hypertrophy. Seven days after the last OVA challenge, untreated mice achieved reduction in airway inflammation, while methacholine maintained the number of BALF total cells, macrophages, and eosinophils. The percentage of goblet cells and the thickness of airway smooth muscle were also maintained by methacholine. Moreover, the treatment of methacholine induced the expression of transforming growth factor (TGF)-β in the lung. This result indicates that the production of TGF-β is involved in induction of airway remodeling caused by bronchoconstriction with methacholine. ***Conclusions:*** Repeated bronchoconstriction caused by methacholine inhalation elicited allergic airway inflammation and airway remodeling.

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Introduction

Bronchial asthma involves allergic airway inflammation, which leads to the release of various inflammatory cytokines and chemokines, resulting in eosinophilic inflammation, goblet cell metaplasia, airway hyperresponsiveness, and thickening of the airway smooth muscle (ASM) [1–3]. Several recent studies have provided some insight into the mechanisms underlying the onset of airway inflammation and remodeling. In general, interleukin (IL)-5 is involved in the proliferation and migration of immature eosinophils in the bone marrow and in the activation of mature eosinophils, whereas IL-4 and IL-13 act on B cells to induce the production of immunoglobulin (Ig)E [4, 5]. Furthermore, transforming growth factor (TGF)-β was shown to contribute to airway remodeling by promoting collagen deposition and thickening of the basement membrane [6–10]. Zhang et al. [11] showed that blocking both IL-13 and IL-25 or their soluble forms of receptors, that is, sIL-13R and sIL-25R, reduced inflammatory cell infiltration, airway hyperresponsiveness, mucus production, and extracellular collagen deposition to achieve overall inhibition of airway remodeling in the murine model of asthma induced by chronic exposure to ovalbumin (OVA).

Airway remodeling in asthma is generated by goblet cell metaplasia, ASM hypertrophy, and subepithelial fibrosis [12]. Grainge et al. [13] showed that repeated events of bronchoconstriction without eosinophilic inflammation caused by provocation with methacholine induced airway remodeling, while provocation with dust mite allergen induced airway remodeling with eosinophilic inflammation.

There are quite a few papers dealing with airway remodeling by focusing on airway inflammation as a cause of remodeling or on biological effects of airway mechanical stress by means of an in vitro system. Such sustained allergic airway inflammation transforms the ASM into a phenotype that facilitates its contraction and expansion, causing repeated constriction and dilation of the airway [14]. This mechanical stress caused by bronchoconstriction imposes a stress for airway epithelial cells, which induces the production of TGF-β that promotes airway remodeling through the generation of collagen synthesis from myofibroblasts [15]. In addition, mechanical stress was found to induce the production of endothelin and TGF-β_2 in normal human bronchial epithelial (NHBE) cells that in turn promoted fibrotic protein synthesis in reporter fibroblasts [16]. Specifically, airway remodeling is generated by fibrosis beneath the basement membrane due to deposition of extracellular matrix (ECM) such as collagen and fibronectin, goblet cell metaplasia, thickening of the ASM, submucosal gland hyperplasia, and angiogenesis [17]. Furthermore, stimulation from epithelial cells and inflammatory cells is considered to induce the production of ECM from fibroblasts and myofibroblasts [18].

These intermittent episodes of compressive stress to NHBE cells under bronchoconstriction were shown to induce goblet cell metaplasia and the expression of mucin (MUC)5AC triggered by TGF-β_2 in vitro [19]. Moreover, mechanical stretching to primary human ASM cells significantly elevated the concentration of adenosine triphosphate (ATP) in cell culture supernatants, which acted as an extracellular mediator in the airway [20]. Collectively, these findings suggest that repeated mechanical stress could also contribute to inducing the airway inflammation as in the case of airway remodeling in asthma.

Several reagents are also known to promote bronchoconstriction, including methacholine, acetylcholine, and histamine [21]. Methacholine is a β-methyl analog of acetylcholine, which has a longer duration of action than acetylcholine, and acts directly on the acetylcholine receptor to cause bronchoconstriction and increase bronchial secretion [21]. The ASM receives direct innervation from the parasympathetic nerve; bronchoconstriction occurs when acetylcholine is released from the nerve endings by stimulation of the vagus nerve, which then acts directly on the acetylcholine receptor [22]. Individuals with asthma are more sensitive to methacholine; therefore, bronchoconstriction occurs with the exposure to a lower dose of inhaled methacholine compared with that induced in a healthy control subject [23]. The intracellular signaling pathway activated by mechanical stress is called mechanotransduction. Tschumperlin et al. [24] reported that pressure and compressive stress on epithelial cells caused mechanotransduction activation and enhanced the production of cytokines and lipid mediators. In addition, Oenema et al. [25] reported that stimulation with methacholine and TGF-β_1 in guinea pigs augmented the expression of contractile proteins such as α-smooth muscle actin (SMA), smooth muscle myosin, and calponin in the lung.

Despite several studies demonstrating that mediators are produced due to mechanical stress, the effects of mechanical stress itself such as physical contraction and expansion of the ASM on the processes of allergic airway inflammation have not been explored. In other words, we raised a question if airway constriction itself could affect not only airway remodeling but also coexisting airway in-

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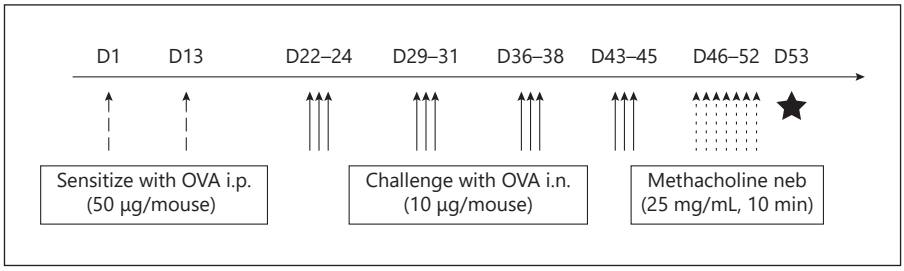

Fig. 1. Protocol for the murine model of OVA-induced allergic airway inflammation and methacholine nebulizer. OVA, ovalbumin.

flammation, which is closer to the natural condition which asthmatics might have. Accordingly, in the present study, we investigated the effect of mechanical stress on allergic airway inflammation using an OVA-induced asthma mouse model with bronchoconstriction caused by inhalation of methacholine. These findings can provide new insight into the underlying mechanism of mechanical stress in maintaining allergic airway inflammation and suggest new strategies for treatment or prevention.

Materials and Methods

Animals and Establishment of the Airway Allergic Inflammation Model

Female BALB/c mice (6–8 weeks old, 19–24 g) were obtained from the Saitama Experimental Animal Supply Co. Ltd. (Saitama, Japan). All animal experiments were approved by the Animal Care and Use Committee of Showa University.

The mice were sensitized with OVA (Sigma-Aldrich, St Louis, MO, USA) emulsified in alum (50 μg/mouse) intraperitoneally on days 1 and 14. The mice were then challenged by intranasal administration of OVA (10 μg/mouse) 3 times a week for 4 weeks from days 22 to 38 (Fig. 1). Methacholine (Sigma-Aldrich, St Louis, MO, USA) was administered to half of the model mice using a nebulizer, at 25 mg/mL for 10 min per day from days 46 to 52. The control mice received only normal saline in the same manner. All experiments were performed at least twice with 4 or more animals per group.

For the additional investigation, the administration of salbutamol (Sigma-Aldrich, St Louis, MO, USA) was performed. Salbutamol was administered by a nebulizer (0.05%, 10 min) twice a day from days 46 to 52 along with administration of methacholine.

Cellular Analysis and Measurement of Cytokines in the Bronchoalveolar Lavage Fluid

The mice were sacrificed 24 h after the last administration of methacholine. The trachea was removed via blunt dissection, and a small caliber needle was inserted and secured in the airway. Three successive washes with 0.35 mL of normal saline were conducted through instillation and then gently aspirated. The recovered bronchoalveolar lavage fluid (BALF) was maintained on ice until centrifugation at 300 *g* for 7 min at 4°C. The obtained cells were then washed again and resuspended in 100 μL of normal saline.

The total number of cells in each BALF sample was calculated using a hemocytometer with Turk solution (Wako, Osaka, Japan).

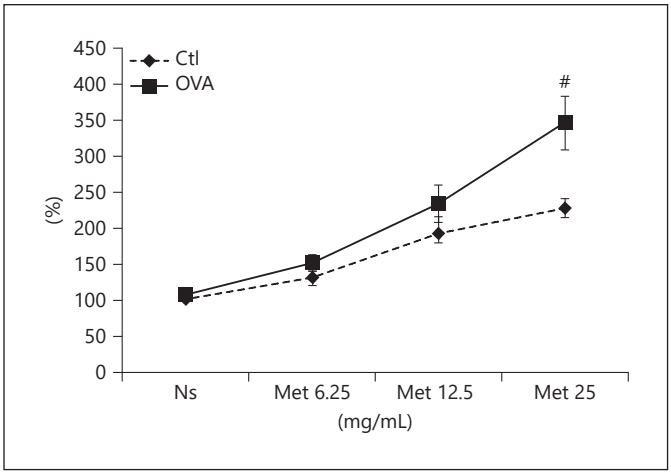

Fig. 2. Airway responsiveness to Met in OVA-induced allergic airway inflammation. The results are expressed as mean ± SEM (n = 5–7). $^{\#}p < 0.05$ compared with Ctl. OVA, ovalbumin; Ctl, control mice; Met, methacholine; Ns, normal saline.

Differential cell counts were performed on cytospin cell preparations (Cytopsin 3; Shandon, Pittsburgh, PA, USA) and stained with Diff-Quik (Dade Behring Inc., Newark, DE, USA). A differential count of 400 cells was performed using standard morphological criteria. Cytokine levels, including IL-4, IL-5, IL-13, and TGF-β, in the BALF were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer instructions.

Lung Histopathology

After collecting the BALF, the lungs were dissected from the chest and inflated with 500 μL of 10% formalin in PBS (pH 7.4) for 24 h at a pressure of 10 cm H_2O. The lung tissues were dehydrated in ethanol, embedded in paraffin, sectioned (5 μm), and stained with periodic acid-Schiff (PAS) stain (Sigma-Aldrich, St Louis, MO, USA). Lung histopathology was then observed under an optical microscope with ×100 magnification. For analysis of goblet cells, 4 lung sections were examined per mouse and the percentage of PAS-positive cells was counted in 4 airways from 1 section. For analysis of smooth muscle hypertrophy, the airways with a diameter of 100–300 μm were randomly selected. The thickness of the ASM was measured from the area of α-SMA-specific immunostaining, and averages of 4 airway walls per animal were calculated. Masson trichrome staining was used for analysis of lung fibrosis.

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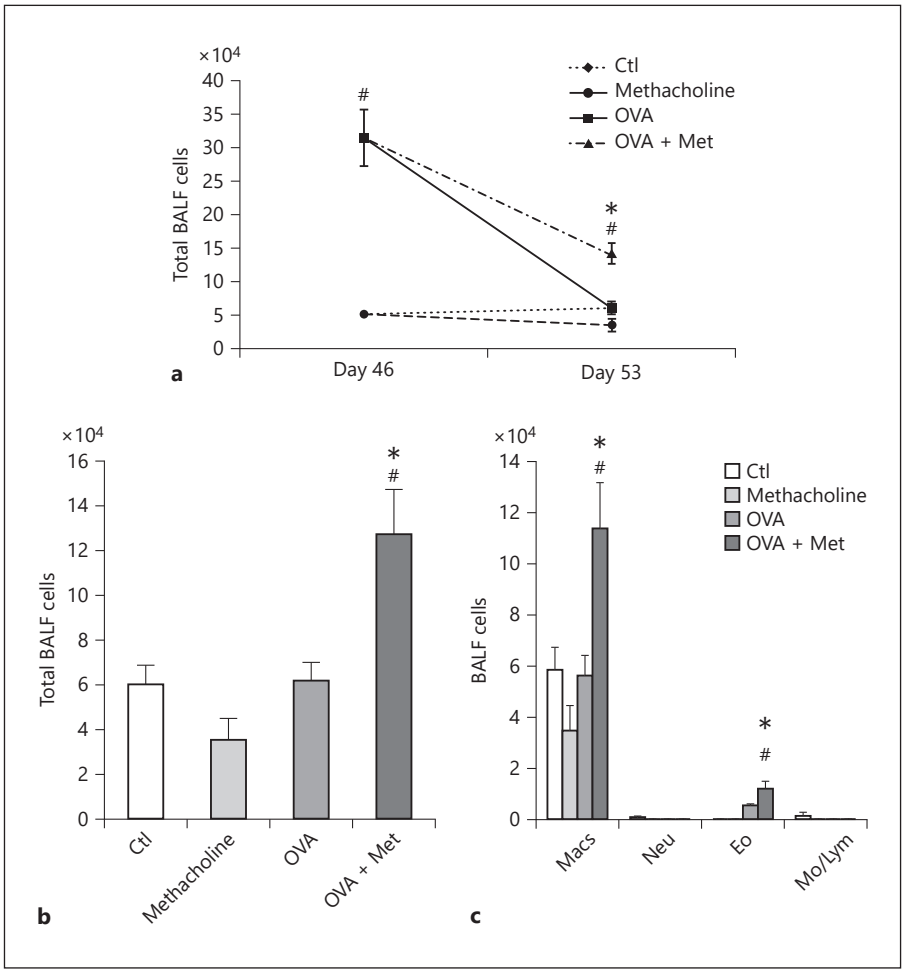

Fig. 3. Analysis of inflammatory cells and cytokines in the murine model of OVA-induced allergic airway inflammation and methacholine nebulizer. Total number of inflammatory cells at days 46 and 53 (**a**), total number of inflammatory cells at day 53 (**b**), and differential count of the inflammatory cells at day 53 (**c**) in the BALF. The results are expressed as mean ± SEM (n = 5–7). * $p < 0.05$ compared with OVA-challenged mice and $^{\#}p < 0.05$ compared with Ctl. OVA, ovalbumin; Ctl, control mice; Met, methacholine; BALF, bronchoalveolar lavage fluid.

The percentage of collagen deposition area in the entire lung field was measured in 3 sections of lungs per mouse by hybrid cell count using a digital microscope (BZ-X800; Keyence, Tokyo, Japan).

Gene Expression in the Lung Tissue

The lung tissue was homogenized, and total RNA was isolated by the RNeasy micro kit (Qiagen, Louisville, KY, USA). The RNA was then reverse transcribed into cDNA with a high-capacity cDNA reverse transcription kit (Thermo Scientific-Applied Biosystems, Foster City, CA, USA). The mRNA levels of *Il-4*, *Il-5*, *Il-13*, and *Tgf-β* were determined by quantitative PCR using SYBR Green/ROX master mix (SABiosciences, Frederick, MD, USA) and primers from SABiosciences. The relative mRNA levels were calculated in reference to the level of *Gapdh* mRNA with the cycle threshold method.

Airway Responsiveness

The airway responsiveness to methacholine was measured on day 46 as previously described [26]. In brief, the mice were tracheostomized and connected to a Harvard ventilator under anesthesia with 50 mg/kg of pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan). The mice were then placed in the plethysmograph chamber (Buxco Electronics Inc., Troy, NY, USA) to measure the degree of airway resistance (Raw). Methacholine was administered at 3 concentrations (6.25, 12.5, and 25 mg/mL) for 3 min using an in-line aerosol system. Data are expressed as the Raw percentage, calculated using the following formula: (Raw after inhalation of methacholine/Raw before inhalation) × 100.

Statistical Analysis

Differences between 2 groups were investigated by an unpaired Student's t test. The significance of the differences between the 3 groups (i.e., control, OVA, and OVA + methacholine [OVA + Met] groups) was determined by analysis of variance with Bonferroni correction. The analyses were performed using the JMP® software (SAS Institute Japan, Tokyo, Japan). Data are expressed as mean ± SEM, and $p < 0.05$ was considered to indicate statistical significance.

Results

Prior to investigating the effect of methacholine in allergic airway inflammation, we compared the murine airway responsiveness to various concentrations of methacholine administered on day 46, 24 h after the last OVA

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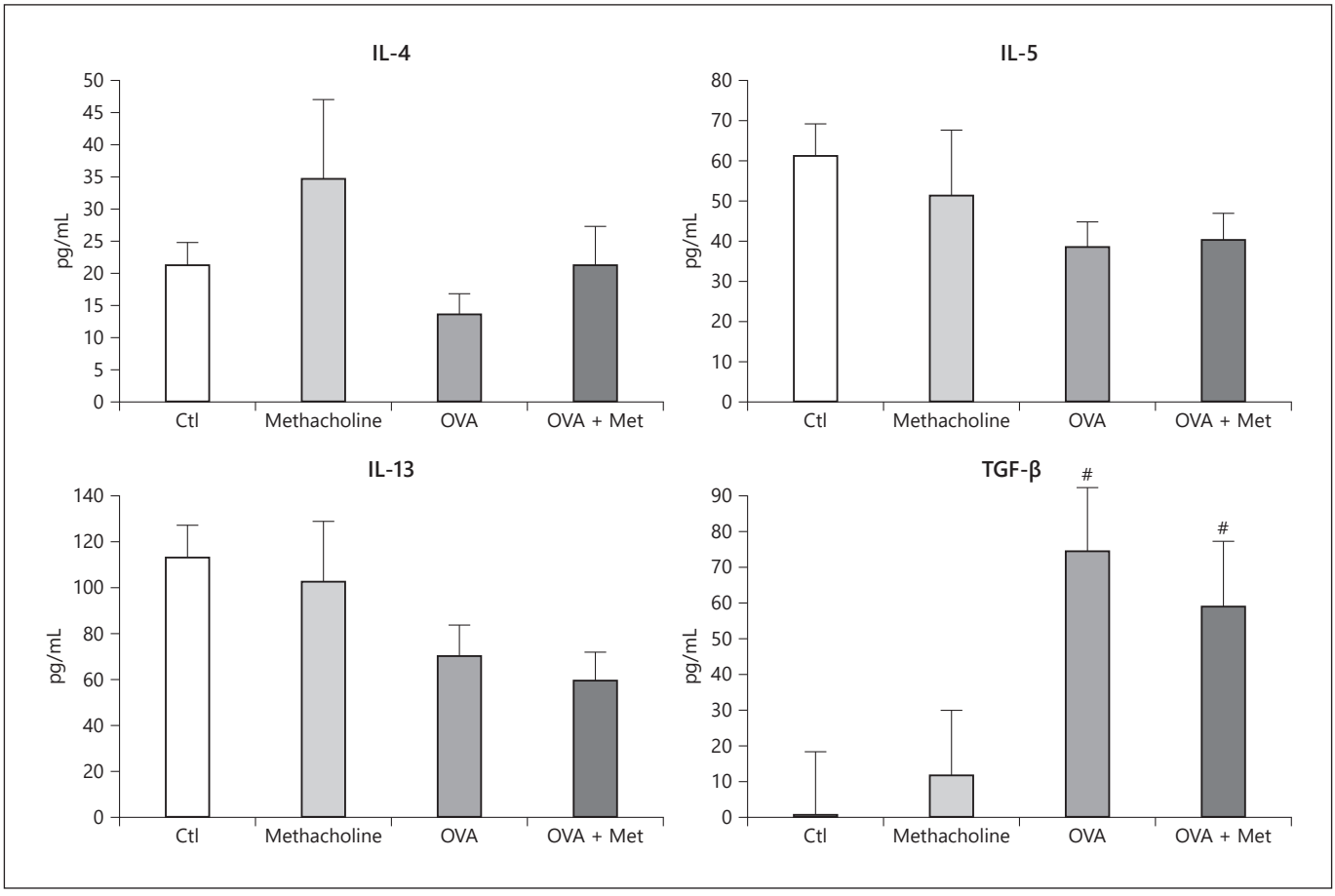

Fig. 4. Analysis of cytokine production in the BALF by ELISA. $^{\#}p < 0.05$ compared with Ctl. BALF, bronchoalveolar lavage fluid; Ctl, control mice; Met, methacholine.

challenge, to determine the appropriate dose that induced adequate bronchoconstriction. The airway responsiveness to methacholine increased in a dose-dependent manner in both the control and OVA + Met groups. Administration of 25 mg/mL of methacholine resulted in significantly higher airway responsiveness in the OVA group compared with that induced in the control group. On the basis of these results, we administered 25 mg/mL of methacholine via a nebulizer in subsequent experiments to best reflect the effect of methacholine on airway inflammation (Fig. 2).

At 24 h after the last OVA challenge (day 46) without methacholine treatment, the number of total cells in the BALF was significantly increased in the OVA group compared with that in the control group (Fig. 3a), and the percentage of eosinophils in the OVA group was 65.5 ± 3.18%. However, these cell numbers all returned to control levels at 1 week after the last OVA challenge (day 53) (Fig. 3b, c). In contrast, 24 h after the last methacholine treatment, the number of total cells, macrophages, and eosinophils was significantly higher in the OVA + Met group compared with that in both the control and OVA groups (Fig. 3b, c). These results demonstrated that the increase in total cells, macrophages, and eosinophils in the BALF caused by OVA sensitization and challenge was maintained by methacholine treatment.

Among the cytokines (IL-4, IL-5, IL-13, and TGF-β) measured in the BALF on day 53, only the level of TGF-β was significantly increased in both OVA and OVA + Met groups compared with that in the control group; however, there was no difference between the OVA group and OVA + Met group (Fig. 4). Since we have previously reported that the levels of cytokines such as IL-4, IL-5, and IL-13 increase at 24 h after the last OVA challenge [27], this result may show that those cytokines have also returned to control levels at 1 week after the last OVA challenge.

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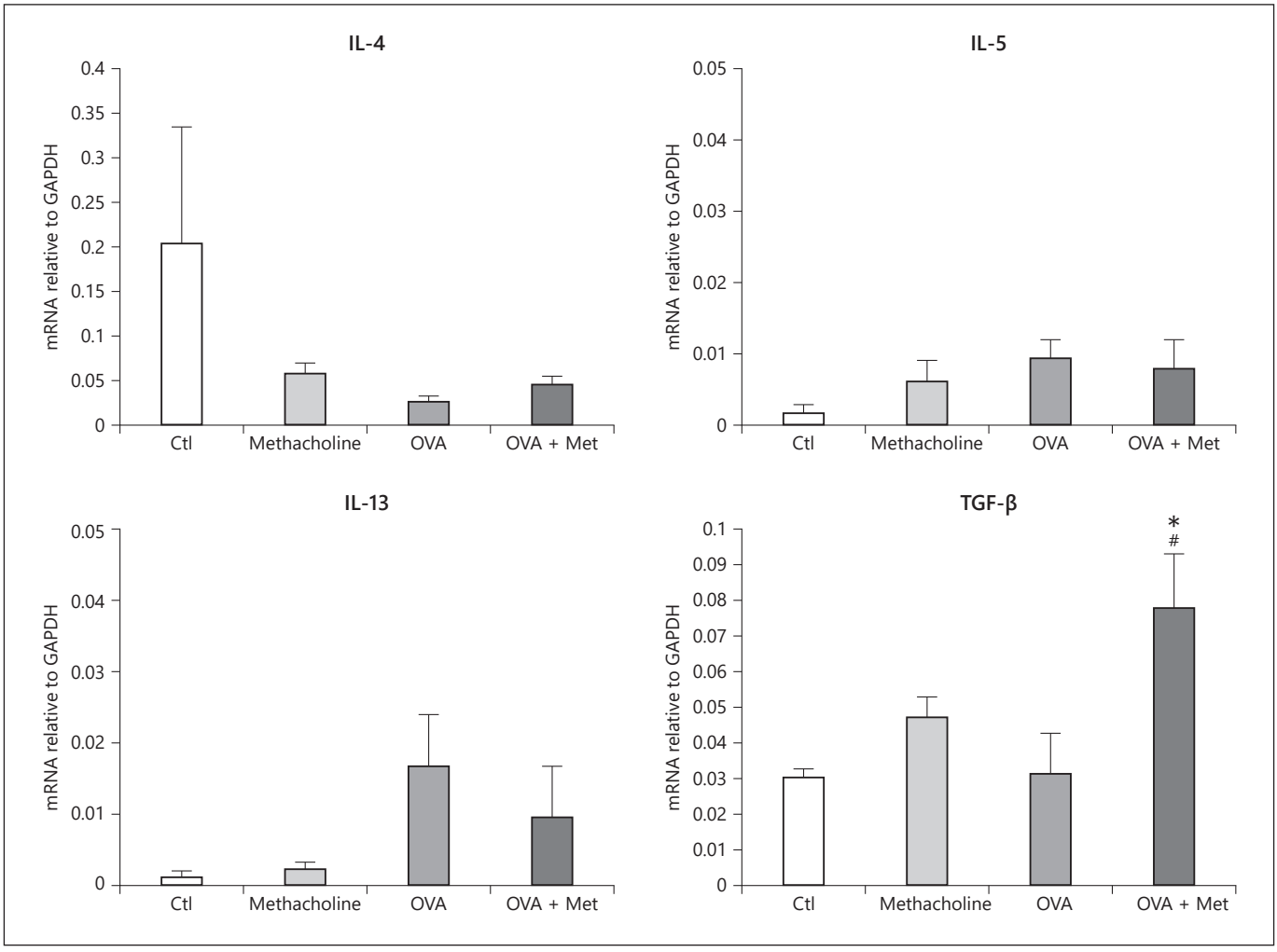

Fig. 5. Analysis of gene expression of cytokines in the lung tissue by qPCR. # $p < 0.05$ compared with Ctl and * $p < 0.05$ compared with OVA-challenged mice. OVA, ovalbumin; Ctl, control mice; Met, methacholine.

At 1 week after the last OVA challenge (day 53), qPCR analysis in the lung showed no difference in the expression level of *Tgf-β* between the control group and OVA group, whereas the expression level of *Tgf-β* was significantly increased in the OVA + Met group compared with that in the other 2 groups (Fig. 5). However, there were no differences in the mRNA levels of other cytokines (*Il-4*, *Il-5*, and *Il-13*) among the 3 groups (Fig. 5).

In terms of the pathological effects of methacholine on the lung tissue at 1 week after the last OVA challenge (day 53), we first quantified the degree of goblet cell metaplasia by counting the percentage of PAS-positive airway epithelial cells. In the OVA group, there was a significant increase in the percentage of PAS-positive cells compared with that in the control group, and a greater proportion of positive cells was found in the OVA + Met group compared with that observed in the OVA group (Fig. 6a). Moreover, there was no difference in the thickness of the ASM between the control group and OVA group, whereas that in the OVA + Met group was significantly higher (Fig. 6b). The percentage of collagen deposition area representing lung fibrosis was enhanced in both the OVA and OVA + Met groups compared with that in the control group, with no difference between the 2 treatment groups (Fig. 6c).

Furthermore, in order to inhibit the bronchoconstriction caused by methacholine, salbutamol, which is a β_2 agonist that acts as a bronchodilator, was administered along with the administration of methacholine. From the investigation, salbutamol administration significantly re-

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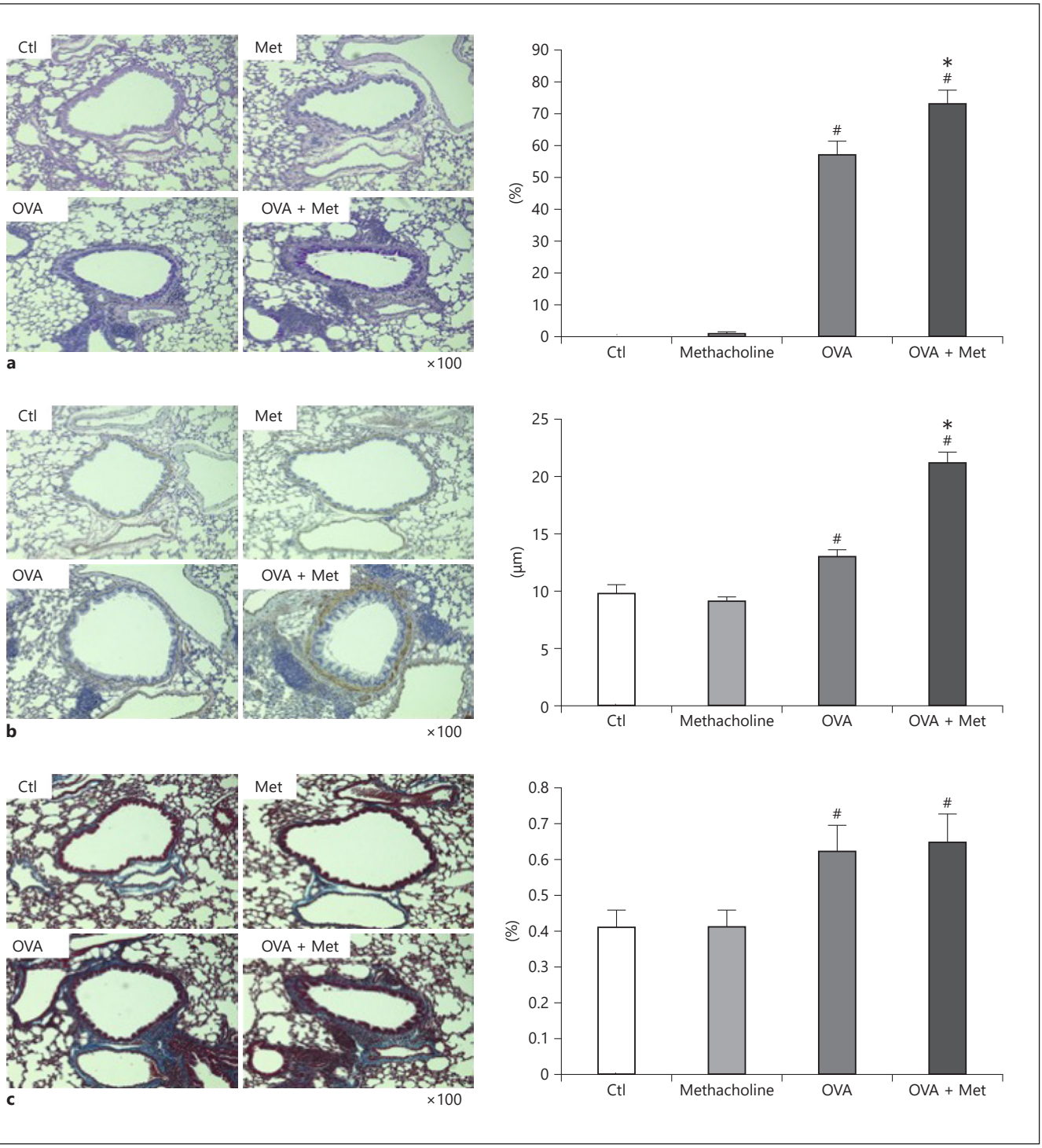

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Fig. 6. Representative lung sections showing PAS staining and statistic analysis for PAS-positive cells (**a**), ASM-specific α-actin staining and statistic analysis for thickness of ASM (**b**), and Masson trichrome staining for analysis of airway fibrosis (**c**) ($n = 5–7$). The results are expressed as mean ± SEM ($n = 5–7$). * $p < 0.05$ compared with OVA-challenged mice and # $p < 0.05$ compared with Ctl. ASM, airway smooth muscle; OVA, ovalbumin; Ctl, control mice; PAS, periodic acid-Schiff; Met, methacholine.

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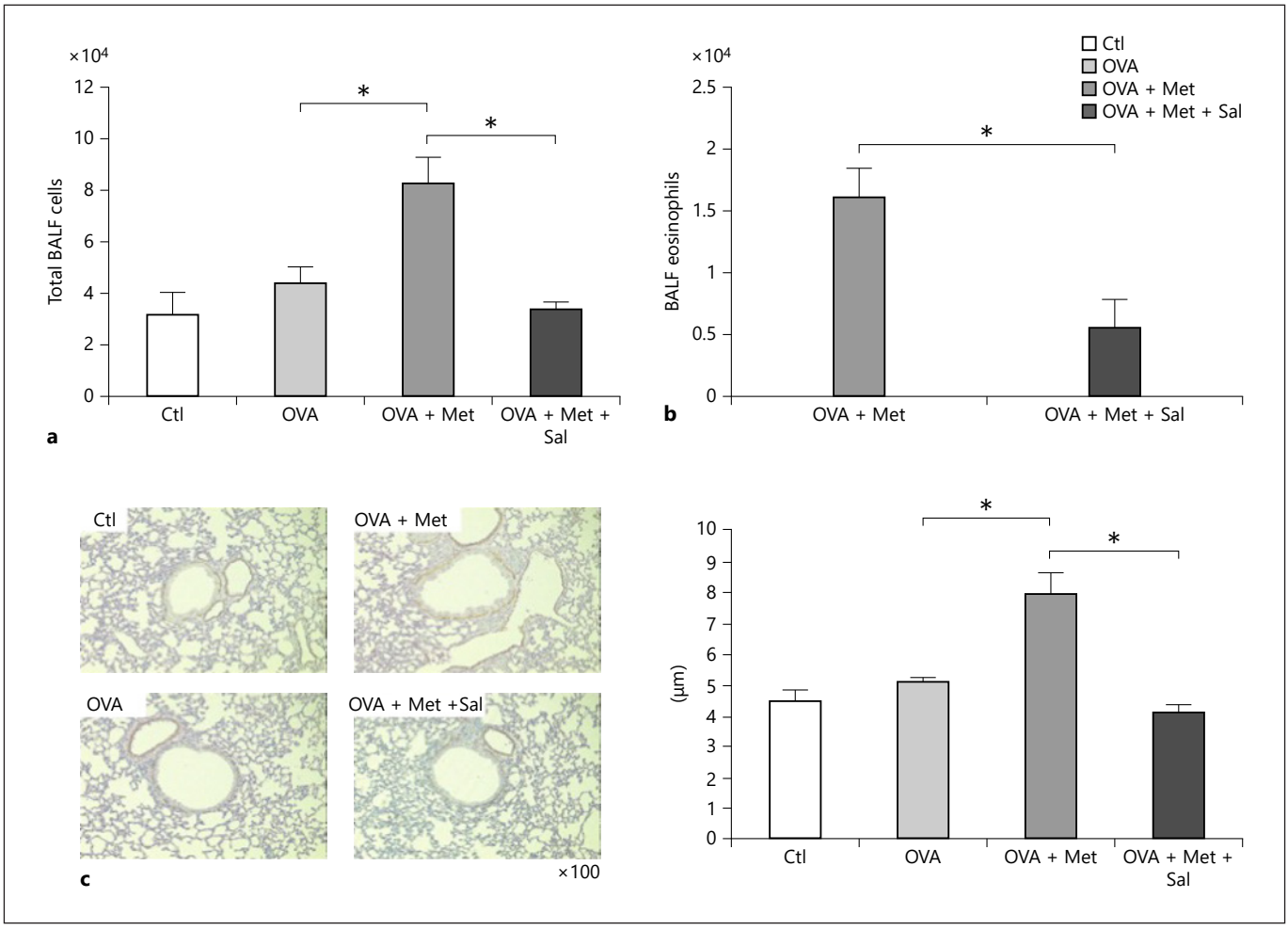

Fig. 7. Analysis of inflammatory cells in the murine model of OVA-induced allergic airway inflammation with methacholine and salbutamol nebulizer. Total number of inflammatory cells (**a**) and the number of eosinophils (**b**). Representative lung sections showing airway smooth muscle-specific α-actin staining and statistic analysis for thickness of airway smooth muscles (**c**). The results are expressed as mean ± SEM ($n = 5–8$). * $p < 0.05$. OVA, ovalbumin; Met, methacholine; BALF, bronchoalveolar lavage fluid; Sal, salbutamol.

duced the number of total cells and eosinophils in the BALF (Fig. 7a, b). In addition, the thickness of ASM was also significantly inhibited by administration of salbutamol compared with the OVA + Met group (Fig. 7c).

Discussion/Conclusion

In the present study, we confirmed that the mechanical stress caused by repeated bronchoconstriction induced by methacholine promotes allergic airway inflammation and remodeling in a mouse model of OVA-induced asthma. The current study confirmed our previous finding that the number of total cells and eosinophils in the BALF was significantly increased and the ASM was significantly thicker at 24 h after the last OVA challenge compared with the control group in a similar murine model of asthma [27]. Importantly, the present study showed that these changes in the OVA group were restored to the levels observed in the control group within 1 week after the last challenge; this indicated that OVA-induced eosinophilic airway inflammation and ASM thickening could be attenuated by the lack of continuous OVA challenge, representing a reversible model of allergic airway inflammation. In contrast, the OVA-challenged mice administered with methacholine showed less attenuation in the number of total cells and eosinophils in the BALF and continued thickening of the ASM even 1 week after the last OVA exposure.

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Moreover, although goblet cell metaplasia was still maintained in the OVA group after 1 week, the degree of metaplasia was significantly greater in the methacholine-administered group. Collectively, these results suggest that the repeated bronchoconstriction caused by methacholine inhalation maintained allergic airway inflammation.

We have considered that the bronchoconstriction caused by methacholine evokes a mechanical stress, which maintained the allergic airway inflammation and induced the remodeling in the OVA + Met group. However, there was still concern that the pharmacological effect of methacholine may contribute to the airway inflammation. To confirm whether the mechanical stress caused by bronchoconstriction affected the airway inflammation, we administered salbutamol along with the administration of methacholine. Salbutamol is a β2-agonist, which acts as a bronchodilator expected to inhibit the bronchoconstriction caused by methacholine and suppress the mechanical stress. From this study, administration of salbutamol inhibited the airway inflammation and remodeling seen in the OVA + Met group. This result suggests that the mechanical stress caused by bronchoconstriction can modulate the airway inflammation and remodeling to be maintained.

Takahara et al. [20] reported that mechanical stress in primary human ASM cells caused the release of ATP from the cells, which increased the concentration of intracellular Ca^{2+} as a mechanism of bronchoconstriction. In addition, Ito et al. [28] found that a novel Ca^{2+} influx pathway was activated by mechanical stress through Ca^{2+} channels, which plays an active role in human ASM cells and is involved in the pathogenesis of asthma. Furthermore, matrix metalloproteinase-9 and -2 enzymes that regulate tissue injury, repair, and remodeling were shown to be produced at higher levels under mechanical stress in NHBE cells [29]. Consistent with these previous findings, thickening of the ASM was observed in the present study as a result of mechanical stress; this might suggest a possibility that the repeated exposure to mechanical stress promotes airway remodeling in asthma. Given the lack of difference in lung fibrosis among the 3 groups, the mechanical stress caused by repeated bronchoconstriction appears to have maintained airway remodeling mainly through enhanced goblet cell metaplasia and thickening of the ASM.

Airway epithelial cells respond to Th2 cytokines and other various inflammatory stimuli, which result in goblet cell metaplasia and production of eotaxin and IL-8. Through this pathway, airway epithelial cells play important roles in the development of allergic airway inflammation and in airway remodeling by producing mediators such as TGF-β and endothelin [30]. Park and Tschumperlin [19] showed that the expression level of MUC5AC was increased under compressive stress in NHBE cells and that the mechanism involved TGF-β2. In line with this previous study, we identified TGF-β as the only cytokine that was maintained in the lung tissue of mice in the OVA + Met group compared with that in the OVA group. The production of TGF-β is generally known to be involved in airway remodeling, and our results indicate that TGF-β has a role in inducing airway remodeling in the lung tissue but not in the alveoli.

Previous reports have demonstrated that salmeterol (long-acting β_2 agonists) and fluticasone (ICS) maintenance therapy could improve asthma control and reduce the risk of future exacerbations compared with the use of fluticasone alone [31]. Moreover, a 3-year investigation comparing the effects of salmeterol/fluticasone, fluticasone alone, and salmeterol alone in patients with asthma showed that salmeterol/fluticasone significantly improved achievement of asthma control, continuous symptoms, respiratory function, and airway hyperresponsiveness compared with the single agents [32]. These results further support our suggestion of early intervention with a bronchodilator along with ICS to inhibit repetitive bronchoconstriction and reduce airway remodeling, which can result in better long-term control of asthma over a long period of time.

The main contribution of this study is the demonstration that the mechanical stress caused by repeated bronchoconstriction maintains eosinophilic airway inflammation and induces airway remodeling. However, a limitation of this study is the possibility that the induction of eosinophils in the BALF and augmentation of airway remodeling might have been caused by the pharmacological action of methacholine rather than the mechanical stress itself. To explore this possibility, it is important to investigate the influence of other agents such as acetylcholine and histamine that cause bronchoconstriction. In addition, further studies are needed to investigate the impact of other physical forces acting on the airway, such as bronchodilation, forced breathing, and frequent cough, which may also cause mechanical stress and thus affect airway inflammation.

In conclusion, we showed that repetitive mechanical bronchoconstriction with methacholine could maintain eosinophilic inflammation and induce airway remodeling in a murine model of asthma. Our findings suggest that the mechanical stress caused by physical forces is associated with repeated bronchoconstriction act on epithelial

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cells and the ASM, which are considered to maintain allergic airway inflammation and to induce remodeling; this implies new pathological mechanisms and treatment strategies to improve long-term asthma control.

Acknowledgement

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Statement of Ethics

All of the animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Showa University (Approval No. 09079), which operated in accordance with the Japanese government guidelines for the care and use of laboratory animals, which adheres to international procedures for animal care.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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The authors did not receive any funding.

Author Contributions

Yoshito Miyata and Shin Ohta designed the study, collected the data, analyzed the data, and wrote the manuscript. Akihiko Tanaka designed the study and revised the manuscript. Ayaka Kashima, Hiromitsu Suganuma, Haruna Sato, Tomoki Uno, Hitomi Ida, Tomoyuki Kimura, Megumi Jinno, and Kuniaki Hirai contributed to data collection. Tetsuya Homma, Mayumi Yamamoto, Yoshio Watanabe, Shintaro Suzuki, and Hironori Sagara designed the study and revised the manuscript. All authors read and approved the final manuscript.

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